Stable overexpression of the type-1 inositol 1,4,5-trisphosphate receptor in L fibroblasts: subcellular distribution and functional consequences

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InsP_3 receptor (InsP_3R)/Ca^{2+}-release channels differ markedly in abundance in different tissues/cell types and InsP_3R expression levels may be modulated in response to a variety of external cues. Cell lines overexpressing InsP_3Rs will provide useful models for the study of the influence of receptor density and subtype on InsP_3-mediated Ca^{2+} signalling. We have investigated the properties of InsP_3Rs in mouse L fibroblast cell lines transfected with either type-1 InsP_3R cDNA (L15) or vector control (Lvec). L15 cells express approximately eightfold higher levels of the type-1 InsP_3R protein than Lvec cells, as assessed by radioligand binding and immunoblotting. Increased expression was stable since it did not alter over ten cell passages. Both L15 and Lvec cells express predominantly the type-1 InsP_3R isoform, indicating that functional differences in the InsP_3-mediated Ca^{2+} signalling in these cell lines are due to alteration in the levels of receptor rather than changes in the isoform expressed. Type-1 InsP_3R in L15 cells is largely associated with subcellular membrane fractions bearing the sarco/endoplasmic reticulum Ca^{2+} ATPase pump, appropriate for rapidly exchanging Ca^{2+} pools. Functionally, there is an approximately fourfold increase in the sensitivity of permeabilized L15-cell Ca^{2+} mobilization in response to increasing concentrations of Ins(1,4,5)P_3. This study indicates that L15/Lvec cells provide a suitable model for studying the effects of InsP_3R expression level on InsP_3-induced Ca^{2+} mobilization.

INTRODUCTION

InsP_3 receptors (InsP_3Rs) act downstream of cell-surface receptors linked to phosphoinositide turnover, mediating Ca^{2+} release from intracellular pools in response to elevations in cytoplasmic InsP_3 concentration (see ref. [1] for review). To date, the complete cDNA sequences of three distinct InsP_3R isoforms (types 1, 2, 3) have been determined [2–4] and partial sequences of candidate fourth and fifth types have been deduced [2,5]. All are very large proteins of calculated molecular masses $\sim$ 305 kDa to $\sim$ 313 kDa, which share 60–70 % amino acid identity between isoforms [1,6,7]. InsP_3-sensitive cation channels are believed to be formed by association of these subunits into tetrameric complexes. InsP_3Rs are predicted to consist of a large cytoplasmic N-terminus, six transmembrane domains and a short cytoplasmic C-terminus. The N-terminal 650 amino acids of the type-1 InsP_3R contains the InsP_3-binding site, separated from the predicted transmembrane/channel domain by a coupling region. This coupling region bears putative sites for the action of various modulators of channel activity, such as Ca^{2+} itself, ATP and phosphorylation by a number of protein kinases [1,6,7].

Various studies indicate that InsP_3-sensitive Ca^{2+} channels are predominantly localized in the endoplasmic reticulum [8,9]. However, in several cell types biochemical and electrophysiological evidence suggests that InsP_3Rs are present in the nuclear envelope [10], plasmalemma [11,12] and invaginations of the plasmalemma, known as cavaeolae [13]. In addition to having multiple subcellular distributions, different InsP_3 isoforms have distinct but overlapping tissue distributions, such that most cell types express more than one isoform simultaneously [14,15]. The type-1 InsP_3R is expressed ubiquitously, although it is 10–100 times more abundant in cerebellum than in peripheral tissues [2]. Within cerebellum, the type-1 InsP_3R is particularly enriched in Purkinje neurons, which display distinct InsP_3-mediated Ca^{2+}-release properties from hepatocytes and glial cells. Purkinje neuron Ca^{2+} mobilization exhibits lower sensitivity to InsP_3 than do glial or peripheral cell types, although the maximal rate of release is considerably higher [16]. Whether the distinct Ca^{2+}-release properties of these neurons result from very high levels of type-1 InsP_3R expression, or from modulation of InsP_3-sensitive Ca^{2+} channels by other factors unique to these cells, remains to be elucidated.

One approach to investigating the effects of different levels of InsP_3R expression on intracellular signalling is the use of cell lines transfected with appropriate cDNAs. Mouse type-1 InsP_3R cDNA isolated from cerebellum has been stably transfected into mouse L fibroblasts [17]. A type-1 InsP_3R-transfected cell-line (L15) showed increased levels of [^{3}H]InsP_3 binding and increased levels of a protein recognized by anti-(type-1 InsP_3R) monoclonal antibodies (mAbs), compared with a vector-transfected control cell line (Lvec). Ca^{2+}-mobilization assays on membrane fractions isolated from these cell lines indicated a large increase in the potency of InsP_3 in eliciting Ca^{2+} release from L15 membranes, and an increase in maximal InsP_3-releasable pool size, compared with Lvec membranes [17].

However, isolated membrane fractions frequently display distinct Ca^{2+}-release properties compared with intact or permeabilized cells [18,19]. The consequences of cellular disruption

Abbreviations used: InsP_3R, InsP_3 receptor; 6-deoxy-InsPS_3, 6-deoxy-D-*myo*-inositol 1,4,5-trisphosphorothioate; 3PS-InsP_3, D-*myo*-inositol 1,4,5-trisphosphate 3-phosphorothioate; gluc(2,3′,4′)P_3, 2-hydroxyethyl α-D-glucopyranoside 2,3′,4′-trisphosphate; DMEM, Dulbecco's modified Eagle's medium; SERCA, sarco/endoplasmic reticulum Ca^{2+} ATPase; mAb, monoclonal antibody; pAb, polyclonal antibody.

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often include a decrease in the size of the maximal InsP_3-releasable Ca^{2+} pool and a decrease in the sensitivity of Ca^{2+} release to InsP_3. In this study, we compared the characteristics of InsP_3-induced Ca^{2+} release in L15 and Lvec cells permeabilized by saponin. The stability of type-1 InsP_3R over expression and the presence of other InsP_3R isoforms were analysed by immunoblotting. Identification of InsP_3R isoforms in control cells is important to confirm that changes in InsP_3-induced Ca^{2+} release are solely due to overexpression of the transfected type-1 InsP_3R cDNA, rather than alteration of the predominant isoform expressed. Stable overexpression of type-1 InsP_3R is a key issue, since stable transfection would permit co-expression of other components of the inositol phosphate signalling system, thereby enhancing detailed understanding of the interactions between these components. Finally, the subcellular localization of type-1 InsP_3R in Lvec and L15 cells was examined using membrane fractionation and immunoblotting techniques, in order to determine if the overexpressed type-1 InsP_3R was present in membrane fractions appropriate for rapid Ca^{2+} uptake and release. These studies indicate that Lvec/L15 cells provide a suitable model for examining the effects of type-1 InsP_3R overexpression on phosphoinositide-linked signal transduction.

EXPERIMENTAL

Materials

$^{45}CaCl_2$, [^{3}H]Ins(1,4,5)P_3 and enhanced chemiluminescence reagents were from Amersham International, Amersham, Bucks, U.K. Disodium ATP, EGTA, saponin and horseradish peroxidase-conjugated secondary antibodies were from Sigma (Poole, Dorset, U.K.). Chemically synthesized Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 were obtained from the University of Rhode Island Foundation Chemistry Group. 2-Hydroxyethyl-α-D-glucopyranoside 2,3′,4′-trisphosphate [gluc(2,3′,4′)P_3], a metabolically resistant adenophostin A and Ins(1,4,5)P_3 analogue, was synthesized and characterized as described [20]. 6-Deoxy-D-*myo*-inositol 1,4,5-trisphosphorothioate (6-deoxy-InsPS_3), a low-intrinsic-activity partial agonist of InsP_3-mediated Ca^{2+} release [21], was generously supplied by Professor B. V. L. Potter (University of Bath, Bath, U.K.). D-*myo*-Inositol 1,4,5-trisphosphate 3-phosphorothioate (3PS-InsP_3), an InsP_4-3-phosphatase-resistant Ins(1,3,4,5)P_4 analogue, was synthesized by Dr. A. Fauq and Professor A. P. Kozikowski (Mayo Clinic, Jacksonville, FL, U.S.A.) as described [22].

mAbs against the type-2 (mAb KM1083) and type-3 (mAb KM1082) InsP_3Rs [14] were generously donated by Dr. M. Hasegawa (Tokyo Research Laboratories, Tokyo, Japan). A mAb (mAb Y1/F4) against sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) [23] was kindly supplied by Dr. F. Michelangeli (University of Birmingham, Birmingham, U.K.). A polyclonal antiserum against a type-1 InsP_3R-specific synthetic peptide was generated and characterized as described previously [24].

Cell culture

Lvec and L15 cells transfected with the pBact-STneoB plasmid alone or a vector containing full-length mouse type-1 InsP_3R cDNA were derived as described [17]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM Hepes supplemented with 10% fetal calf serum, 50 units/ml penicillin G, 50 μg/ml streptomycin and 2.5 μg/ml fungizone, in humidified 95% air/5% CO_2. Medium was changed daily, and confluent flasks were harvested using sterile PBS/EDTA, pelleted at 500 g_{max} for 5 min, then reseeded at a density of 2×10^4 cells/cm^2. SH-SY5Y human neuroblastoma cells were grown as described previously [25]. PC-12 rat adrenal pheochromocytoma cells were cultured in DMEM containing 5% fetal calf serum, 10% horse serum and the antibiotics listed above.

Subcellular fractionation

Crude microsomal membrane fractions were prepared from confluent monolayers of Lvec, L15, PC-12 or SH-SY5Y cells grown in eight 175 cm^2 flasks, using the protocol described by Chen et al. [26]. All steps were performed at 4 °C. In brief, cell layers were washed with 2×10 ml of ice-cold PBS containing protease inhibitors (0.5 mM PMSF, 2 mM iodoacetamide, 1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A, 1 μg/ml aprotinin) and 5 mM EDTA, then harvested by agitation in a further 10 ml of this buffer. Cells were pelleted at 12000 g_{max} for 10 min, resuspended in 10 ml of PBS and protease inhibitors, then repelleted. They were then homogenized in 4 ml of 0.5 mM $MgCl_2$/25 mM Tris/Hepes, pH 7.4, containing protease inhibitors by 50 strokes of a glass/Teflon homogenizer. An equal volume of buffer consisting of 0.3 M KCl, 0.5 M sucrose, 40 μM $CaCl_2$ and 25 mM Tris/Hepes, pH 7.4, and containing protease inhibitors was added, followed by a further 25 homogenization strokes. The homogenate was centrifuged at 4300 g_{max} for 20 min and the resulting supernatant centrifuged at 100000 g_{max} for 90 min. The crude microsomal pellet was resuspended in either buffer consisting of 0.3 M sucrose, 0.15 M KCl, 20 μM $CaCl_2$ and 25 mM Tris/Hepes, pH 7.4, and containing protease inhibitors, or buffer A as described below.

Microsomal membranes were further subfractionated using a modification of the procedure of Nigam and Blobel [27]. In brief, crude microsomes were resuspended in 8 ml of buffer A (0.3 M sucrose, 15 mM CsCl, 0.2 mM PMSF, 25 mM Tris/Hepes, pH 7.4) by 20 passes of a loose-fitting glass/Teflon homogenizer. This material was carefully layered on to 12 ml of 1.3 M sucrose in buffer A, then centrifuged at 100000 g_{max} for 90 min at 4 °C (Beckman Ti70 rotor). Four fractions were collected: an interphase (fraction II), the 0.3 M (fraction I) and 1.3 M (fraction III) sucrose layers, and a pellet (fraction IV). Fractions were diluted at least fivefold in buffer A without sucrose and CsCl, then pelleted at 100000 g_{max} for 60 min at 4 °C. Pellets were resuspended in 250 μl of buffer containing 0.3 M sucrose, 0.15 M KCl, 20 μM $CaCl_2$, 0.2 mM PMSF and 25 mM Tris/Hepes, pH 7.4, by ten passes through a narrow-gauge needle. Resuspended membranes were frozen on solid CO_2, then stored at −80 °C until use.

SDS/PAGE and immunoblot analysis

Crude microsomal membrane or membrane subfraction protein (20 μg) was resolved on SDS/PAGE minigels (5% polyacrylamide), then transferred on to nitrocellulose using semi-dry apparatus at 2 mA/cm^2 constant current for 2 h in a buffer consisting of 150 mM glycine, 20 mM Tris and 0.037% SDS. Blots were blocked overnight in 5% non-fat milk/PBS, then incubated with primary antibodies for 2 h. These were either CT-1, a rabbit polyclonal antiserum raised against a C-terminal peptide of type-1 InsP_3R [24] diluted 1:2000, KM1083 and KM1082, mouse mAbs against C-terminal peptides from the type-2 and type-3 IP_3Rs respectively [14] diluted 1:250, or Y1/F4, a mouse mAb which recognizes SERCA Ca^{2+} pumps [23] diluted 1:200 in PBS/milk. Blots were washed with three changes of PBS over 30 min, then incubated in a 1:1000 dilution of either goat anti-rabbit or rabbit anti-mouse IgG horseradish peroxidase

conjugate for 1 h. After a further three washes in PBS over 30 min, immunoreactive proteins were detected using enhanced chemiluminescence reagents. Band densities of immunoreactive proteins were quantified by scanning densitometry (Bio-Rad model GS-670 scanning densitometer).

Ins(1,4,5)P_3-binding assay

A membrane fraction of either Lvec or L15 cells was recovered for characterization of Ins(1,4,5)P_3-binding sites essentially as described previously [28]. In brief, four to six confluent monolayers of Lvec or L15 cells grown in 175 cm^2 flasks were harvested in PBS/EDTA and pelleted by centrifugation. Pellets were homogenized in ice-cold 20 mM $NaHCO_3$/1 mM dithiothreitol, pH 8.0, and a membrane pellet was recovered by centrifugation (40000 g_{max}; 20 min; 4 °C). The pellet was washed twice by rehomogenization in the same solution and centrifugation. The final membrane fraction was resuspended by homogenization and adjusted to 5–6 mg of protein/ml before immediate storage at −80 °C.

The ability of D-Ins(1,4,5)P_3, L-Ins(1,4,5)P_3 and D-Ins(1,3,4,5)P_4 to displace specific [^{3}H]Ins(1,4,5)P_3 binding from either Lvec or L15 membranes was assessed as described previously [28]. In brief, increasing concentrations of D-Ins(1,4,5)P_3 (1–1000 nM), L-Ins(1,4,5)P_3 (1–50 μM) and D-Ins(1,3,4,5)P_4 (10–10000 nM) were incubated in a total volume of 120 μl with 0.9 nM [^{3}H]Ins(1,4,5)P_3 (~ 8000 d.p.m./assay) in a buffer containing 25 mM Tris/HCl, 5 mM $NaHCO_3$, 1 mM EDTA and 0.25 mM dithiothreitol, pH 8.0. Assays were initiated by addition of 30 μl of the membrane preparation (containing 200–250 or 100–150 μg of protein for Lvec and L15 membranes respectively) and continued for 30 min at 4 °C with intermittent vortex-mixing. Bound and free fractions were separated by rapid vacuum filtration on GF/B filter disks and washing with 3 × 3 ml of 25 mM Tris/HCl, pH 8.0, containing 5 mM $NaHCO_3$ and 1 mM EDTA. In all experiments, 10 μM Ins(1,4,5)P_3 or 100 μg/ml low-molecular-mass heparin defined a similar non-specific binding component.

$^{45}Ca^{2+}$-mobilization assay

$^{45}Ca^{2+}$ mobilization was assessed in saponin-permeabilized L15 and Lvec cells at 20–22 °C, or at 4 °C to limit inositol phosphate metabolism, as described previously [25,29]. Cells were generally saponin-permeabilized in suspension, but for some experiments, adherent permeabilized cells were utilized. For Ins(1,3,4,5)P_4-induced Ca^{2+}-release experiments, InsP_6 (10 μM) was included in the cytosol-like buffer (CLB) to suppress Ins(1,3,4,5)P_4-3-phosphatase activity [29]. Assays were performed in duplicate for each experiment. Ionomycin (5 μM, free acid) was used to define the total releasable $^{45}Ca^{2+}$ pool, and Ins(1,4,5)P_3 (20–30 μM) to define the Ins(1,4,5)P_3-sensitive pool.

RESULTS

Characterization of InsP_3R isoforms expressed in Lvec and L15 cells

Antibodies raised against isoform-specific synthetic peptides from types-1, -2 and -3 InsP_3R [14,24] were used to identify the isoforms of these Ca^{2+}-release channel proteins in Lvec and L15 cells. Lvec cells endogenously express an anti-(type-1 InsP_3R) serum immunoreactive protein at low levels, which has an apparent molecular mass of ~ 260 kDa (Figure 1). This protein is of slightly higher electrophoretic mobility than the type-1 InsP_3R expressed in L15 cells, transfected with full-length mouse

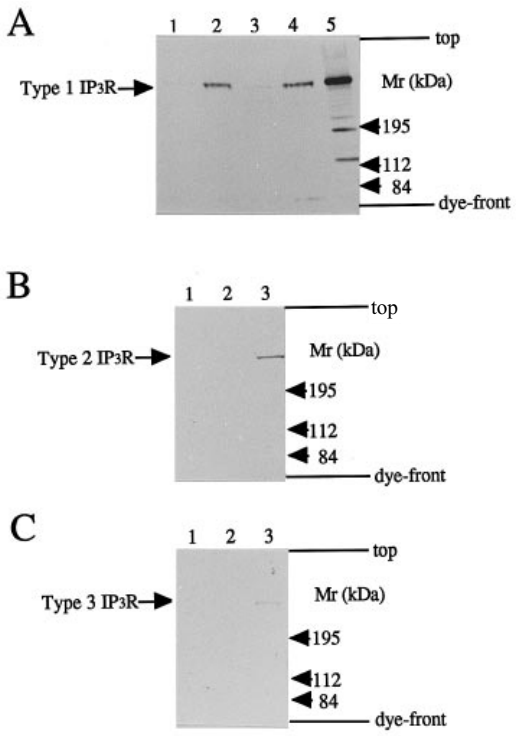

Figure 1 Biochemical characterization of InsP_3R isoforms expressed in Lvec and L15 cells

(**A**) Stable overexpression of the type-1 InsP_3R in L15 cells. L15 cell microsomes (lanes 2 and 4; 20 μg of protein/lane) display 8.5(±0.9)-fold higher expression of type-1 InsP_3R protein than Lvec cell microsomes (lanes 1 and 3), as assessed using scanning densitometry of immunoblots probed with rabbit polyclonal antiserum CT-1. Overexpression is stable, since there is no change in relative abundance of the type-1 InsP_3R between cell passages 8 (lanes 1 and 2) and 18 (lanes 3 and 4) for both cell lines. Overexpressed type-1 InsP_3R protein displays similar electrophoretic mobility to that in rat cerebellar microsomes (lane 5). (**B**) Lvec and L15 cells express barely detectable levels of type-2 InsP_3R. SH-SY5Y human neuroblastoma microsomes (lane 3; 20 μg of protein) contain a protein of apparent molecular mass ~ 250 kDa which is recognized by a mAb (KM1083) raised against a type-2 InsP_3R-specific synthetic peptide. mAb KM1083 could only detect type-2 InsP_3R in Lvec and L15 cells (lanes 1 and 2 respectively; 20 μg of protein/lane) if twice the amount of microsomal protein (40 μg) was loaded on to SDS/polyacrylamide gels and the blot exposed for 4 times as long as for the SH-SY5Y control. (**C**) Type-3 InsP_3R is undetectable in Lvec/L15 cells. Although mAb KM1082, raised against a type-3 InsP_3R-specific peptide, recognized a protein of ~ 250 kDa in PC-12 cell microsomes (lane 3), no immunoreactive proteins could be detected in Lvec (lane 1)/L15 (lane 2) microsomal preparations (all loaded at 20 μg of protein/lane). In all parts, molecular-mass values are indicated in kDa.

type-1 InsP_3R cDNA, or that expressed in rodent cerebellum (Figure 1A and [17]). Bands in rat cerebellum recognized by polyclonal antibody (pAb) CT-1, which are of higher electrophoretic mobility than the type-1 InsP_3R, probably represent products of its proteolytic degradation, since their relative abundance is dramatically increased in microsomes prepared without protease inhibitors. Levels of expression of the type-1 InsP_3R are 8.5(±0.9, $n = 3$)-fold greater in L15 than in Lvec cells, as assessed by densitometry of immunoblots. This approach is semiquantitative, since there was a linear relationship between signal density and the amount of cerebellar microsomal protein loaded over the range at which L15 and Lvec cells expressed type-1 InsP_3R. Cerebellum is a useful standard for quantification of the type-1 InsP_3R, since this isoform is predominantly expressed [5,15]. Figure 1(A) indicates that overexpression of type-1 InsP_3R protein, as assessed by immunoblot analysis, is

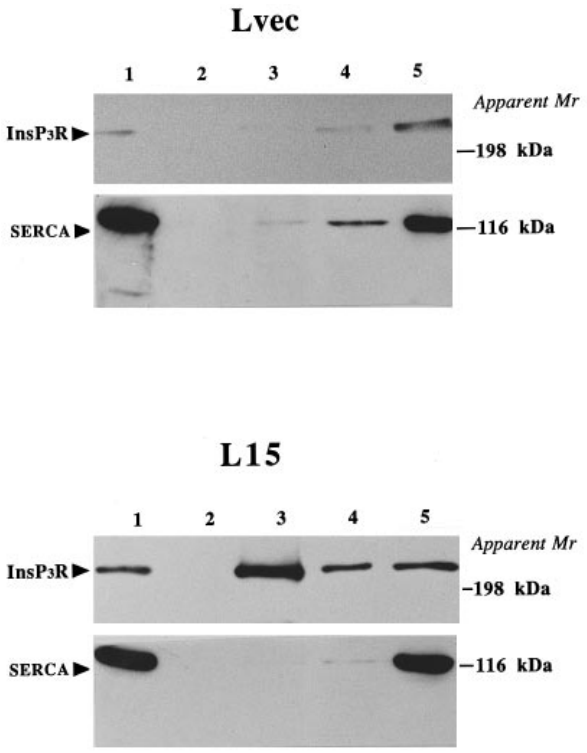

Figure 2 Subcellular distribution of type-1 InsP_3R in Lvec and L15 cells

Lvec and L15 cells display similar subcellular distribution of SERCA proteins in membrane subfractions (fractions I–IV, lanes 2–5) prepared from crude microsomes (lane 1), detected using mAb Y1/F4. SERCA pumps were at greatest relative abundance in the densest membranes (fraction IV, lane 5) and were undetectable in the lightest (fraction I, lane 2), in both L15 and Lvec cells. In Lvec cells, the subcellular distribution of endogenously expressed type-1 InsP_3R mirrored that of the SERCA pump. In L15 cells, type-1 InsP_3R was also restricted to membranes bearing the SERCA pump, although it was at greatest relative abundance in the second lightest fraction (fraction II, lane 3), which contained a barely detectable quantity of this ATPase. However, fraction IV bears the greatest quantity of SERCA pump, the greatest quantity of total protein and the greatest absolute quantity of the type-1 InsP_3R (Table 1) in L15 cells. This distribution pattern represents the mean (±S.E.M.) of three separate experiments. All proteins were loaded at 20 μg/lane; apparent molecular masses indicated are those of Sigma prestained standards.

Table 1 Recovery of protein and type-1 InsP_3R in membrane subfractions of L15 cell microsomes

The total protein and InsP_3R content of microsomal membrane subfractions are shown. Percentage of microsomal InsP_3R was determined by scanning densitometry of immunoblots, as described in the Experimental section. Values represent the mean ± S.E.M. of three separate experiments. N.D., not detectable.

Membrane fraction	Protein (mg)	Percentage of microsomal InsP_3R
Microsomes	4.38 ± 0.33	100
I	0.12 ± 0.04	N.D.
II	0.35 ± 0.04	19.4 ± 5.4
III	0.73 ± 0.04	10.9 ± 1.9
IV	2.45 ± 0.62	69.6 ± 1.8

stable for at least ten cell passages. Further immunoblot studies have indicated that overexpression is stable for at least 30 passages.

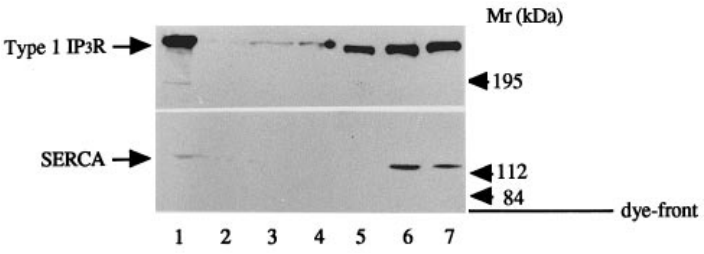

Figure 3 Subfractionation of SH-SY5Y cell microsomal membranes

SH-SY5Y cell microsomes were subfractionated on a sucrose cushion, then membrane proteins (20 μg/lane) were resolved by SDS/PAGE, transferred to nitrocellulose and probed using mAb Y1/F4 or pAb CT-1 to detect SERCA pumps or the type-1 InsP_3R. Samples loaded are rat cerebellar microsomes (lane 1), and SH-SY5Y crude homogenate (lane 2), crude microsomes (lane 3), or sucrose cushion subfractions I to IV (lanes 4–7). Apparent molecular masses (given in kDa) are those of Sigma high-molecular-mass prestained standards. The dye front is at the bottom.

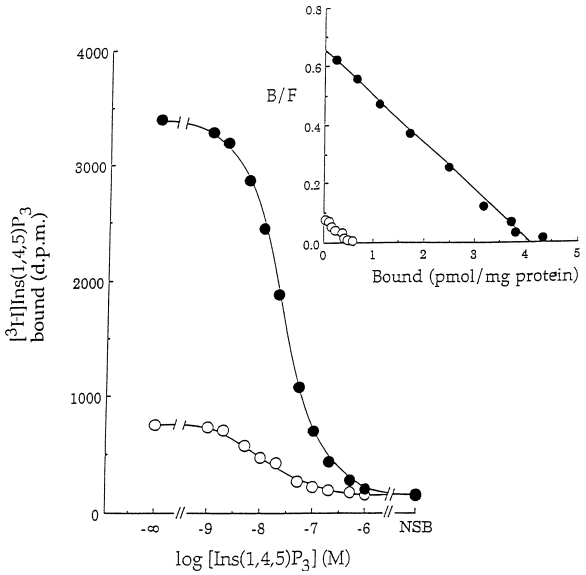

Figure 4 [^{3}H]Ins(1,4,5)P_3 binding to Lvec and L15 cell membranes

A representative experiment where Lvec (open symbols) or L15 (closed symbols) membranes were incubated with 0.9 nM [^{3}H]Ins(1,4,5)P_3 and increasing concentrations of Ins(1,4,5)P_3. Non-specific binding (NSB) was determined in the presence of 10 μM Ins(1,4,5)P_3. The inset shows a Scatchard transformation of the isotope dilution data sets. In this experiment L15 membranes (B_{max} 4150 fmol/mg of protein) exhibited a 7.3-fold higher density of Ins(1,4,5)P_3-binding sites than Lvec membranes (B_{max} 569 fmol/mg) with no significant difference in affinity (K_d values 11.2 nM and 12.1 nM respectively).

Lvec and L15 cells do not express the type-3 InsP_3R, as assessed using mAb KM1082 on immunoblots, with PC-12 cell microsomes as a positive control [5,15]. mAb KM1083, raised against a type-2 InsP_3R-specific peptide, recognized a protein of apparent molecular mass ~ 250 kDa in microsomes prepared from human SH-SY5Y neuroblastoma cells. Type-2 InsP_3R was only detected in L15/Lvec cell microsomes when twice the amount of protein, twice the antibody concentration and a fourfold longer exposure time were employed. In addition, the expression of type-2 InsP_3R does not appear to be altered by overexpression of type-1 InsP_3R in L15 compared with Lvec cells.

Table 2 Inositol polyphosphate selectivity of Lvec and L15 D-Ins(1,4,5)P_3 binding sites

All values are means ± S.E.M. for at least 3 determinations. All slope factors for displacement isotherms were not significantly different to unity. No significant change in either the B_{max} or K_d for Ins(1,4,5)P_3 binding to L15 or Lvec membranes was observed over ten cell passages.

	IC_{50} values	
	Lvec	L15
D-Ins(1,4,5)P_3	$1.46(\pm 0.13) \times 10^{-8}$ M	$1.37(\pm 0.30) \times 10^{-8}$ M
L-Ins(1,4,5)P_3	$2.17(\pm 0.81) \times 10^{-5}$ M	$2.55(\pm 0.44) \times 10^{-5}$ M
D-Ins(1,3,4,5)P_4	$9.88(\pm 0.79) \times 10^{-7}$ M	$8.61(\pm 1.01) \times 10^{-7}$ M

The subcellular distribution of type-1 InsP_3R in Lvec and L15 cells was characterized by immunoblotting of membrane fractions prepared by centrifugation of crude microsomal membranes on a sucrose step-gradient. A mAb which recognizes members of the SERCA family of intracellular Ca^{2+} pump was used as a marker for membranes with potential for rapid Ca^{2+} accumulation (Figure 2). The SERCA pump displayed a similar subcellular distribution in both cell types, being greatly enriched in the most dense membrane fraction (fraction IV) and undetectable in the least dense (fraction I). In Lvec cells, the distribution of endogenously expressed type-1 InsP_3R mirrored that of the SERCA protein. Similarly, in L15 cells, type-1 InsP_3R protein was only present in membrane fractions containing SERCA pumps; however, it was most highly enriched in a fraction containing a barely detectable amount of the SERCA protein (fraction II). Again, both Ca^{2+} pumps and Ca^{2+}-release channels are absent from the lightest L15 cell membranes (fraction I). However, fraction II represents a small proportion of the total microsomal protein (Table 1). When expressed as a percentage of the total microsomal InsP_3R (i.e. InsP_3R band density × total protein of each subfraction), fraction IV contains the greatest overall amount of InsP_3R (Table 1). Furthermore, a similar subcellular distribution of type-1 InsP_3R and SERCA pumps is shown in SH-SY5Y neuroblastoma membranes, which endogenously express high levels of this Ca^{2+}-release channel. In SH-SY5Y cells, SERCA pumps are present at greatest relative abundance in fractions III and IV, although the type-1 InsP_3R is evenly distributed between fractions II and IV (Figure 3, lanes 5–7).

Binding of inositol polyphosphates to Lvec and L15 membranes

In addition to stable overexpression of type-1 InsP_3 protein, L15 cells exhibited stably increased levels of [^{3}H]Ins(1,4,5)P_3 binding. Figure 4 shows a representative experiment of isotope-dilution curves and their Scatchard transformations for [^{3}H]Ins(1,4,5)P_3 binding to Lvec and L15 membranes. Overall L15 cells exhibited an 8.3 (±0.8)-fold greater density of [^{3}H]Ins(1,4,5)P_3-binding sites than Lvec membranes [B_{max} values L15, 3560 ± 280 ($n = 4$); Lvec, 440 ± 50 fmol/mg of protein ($n = 4$)], while no difference in binding affinities was apparent (K_d values 10.2 ± 1.9 and 10.7 ± 1.4 nM for L15 and Lvec membranes respectively). The EC_{50} values for the displacement of [^{3}H]Ins(1,4,5)P_3 from Lvec and L15 cell membranes by D-Ins(1,4,5)P_3, L-Ins(1,4,5)P_3 and D-Ins(1,3,4,5)P_4 are summarized in Table 2. No significant change in either the B_{max} or K_d for D-Ins(1,4,5)P_3 binding to L15 or Lvec membranes was observed over ten cell passages.

Inositol polyphosphate-mediated Ca^{2+} release in Lvec and L15 cells

Since preliminary studies failed to identify phosphoinositide-turnover-linked receptors at the surface of Lvec cells, the effects of overexpression of the type-1 InsP_3R on Ins(1,4,5)P_3-mediated Ca^{2+} mobilization were investigated using permeabilized cell systems. L15 cells permeabilized in suspension exhibited a lower EC_{50} for InsP_3-induced Ca^{2+} release than did Lvec cells under the same conditions (EC_{50} values: Lvec, 176 nM; L15, 45 nM; see Table 3). In addition, L15 cells display a slightly, but significantly, larger InsP_3-releasable Ca^{2+} pool than do Lvec cells [74.1 ± 0.2 % of the total mobilizable pool (determined using ionomycin) compared with 68.1 ± 3.8 % of the total] (Figure 5). This difference cannot be accounted for in terms of the sizes of the ionomycin-sensitive Ca^{2+} pool, since they were indistinguishable in these two cell lines. In adherent permeabilized cells, the

Table 3 Potencies of various inositol polyphosphate analogues at releasing $^{45}Ca^{2+}$ from suspension-permeabilized Lvec and L15 cells

EC_{50} values for $^{45}Ca^{2+}$ release were determined using steady-state assays on suspension-permeabilized cells at either 4 °C or room temperature (RT, 20–22 °C). Differences between L15 and Lvec cell log EC_{50} values were tested for significance using Student's t-test ([a]$P < 0.0001$; [b]$P < 0.001$; [c]$P < 0.05$). All mean values were derived from four separate experiments. Results for $\log_{10}$ EC_{50} value are means ± S.E.M.

	L15 cells		Lvec cells		
	Mean EC_{50} (μM)	Mean $\log_{10}$ EC_{50} (M)	Mean EC_{50} (μM)	Mean $\log_{10}$ EC_{50} (M)	Ratio EC_{50} L15/EC_{50} Lvec
Ins(1,4,5)P_3					
RT	0.045	−7.352 ± 0.041	0.176	−6.754 ± 0.027	3.911[a]
4 °C	0.043	−7.371 ± 0.034	0.160	−6.797 ± 0.035	3.721[a]
3PS-Ins(1,4,5)P_3					
RT	0.7	−6.150 ± 0.02	2.3	−5.635 ± 0.073	3.286[b]
4 °C	1.9	−5.714 ± 0.025	4.1	−5.385 ± 0.031	2.158[b]
6-Deoxy-Ins(1,4,5)PS_3					
RT	8.4	−5.078 ± 0.064	19.2	−4.718 ± 0.088	2.286[c]
Gluc(2,3′,4′)P_3					
RT	0.563	−6.249 ± 0.033	1.566	−5.805 ± 0.014	2.778[a]

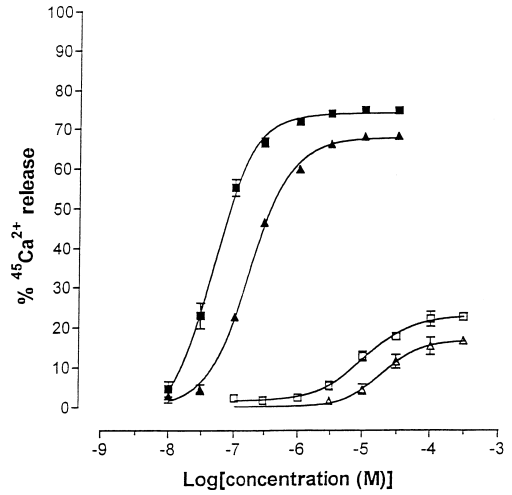

Figure 5 Dose–response curves of Ca^{2+} mobilization in permeabilized Lvec and L15 cells by Ins(1,4,5)P_3 and the partial agonist 6-deoxy-Ins(1,4,5)PS_3

Dose–response relationships for Ins(1,4,5)P_3 (filled symbols) or the partial agonist 6-deoxy-Ins(1,4,5)PS_3 (open symbols) for $^{45}Ca^{2+}$ mobilization from suspension-permeabilized L15 (□, ■) or Lvec (△, ▲) cells. Assays were performed at room temperature (20–22 °C) and results are expressed as a percentage of the total ionomycin-releasable Ca^{2+} pool. Data points represent the mean ± S.E.M. for four separate experiments.

increased sensitivity of L15 cells to InsP_3-induced Ca^{2+} release (EC_{50} 165 nM) relative to that of Lvec cells (EC_{50} 311 nM) was retained, although there was no significant difference in the extent of the InsP_3-releasable pool (L15, 67.0 ± 3.8 %; Lvec, 67.5 ± 1.1 % of the ionomycin releasable pool).

Ins(1,3,4,5)P_4 displayed a similar shift in potency of releasing $^{45}Ca^{2+}$ from Lvec and L15 cells (Figure 5 and Table 3). Use of the metabolism-resistant Ins(1,4,5)P_3 [Gluc(2,3′,4′)P_3] and Ins(1,3,4,5)P_4 (3PS-InsP_3) analogues, or performing assays at 4 °C rather than at room temperature, also demonstrated a 2–4-fold increase in potency in L15 compared with Lvec cells (Table 3). This suggests that the observed difference in potency of various inositol polyphosphates and synthetic analogues at eliciting Ca^{2+} release is a consequence of overexpression of InsP_3-activated channels rather than any differences in inositol phosphate metabolism between these cell lines.

As expected, the partial agonist 6-deoxy-InsPS_3 [21] failed to release the entire Lvec or L15 Ins(1,4,5)P_3-sensitive Ca^{2+} pool at a maximally effective (300 μM) concentration (Figure 5). The shift in the concentration–response curve of Ca^{2+} mobilization by 6-deoxy-InsPS_3 between Lvec and L15 cells remained constant over the entire concentration range used, as is observed for the full agonist Ins(1,4,5)P_3 (Figure 5).

Preliminary experiments have indicated the absence of cell-surface receptors linked to phosphoinositide turnover in Lvec/L15 cells, stimulated using a wide variety of agonists (angiotensin II, ATP, UTP, endothelin, bradykinin, platelet-activating factor, 5-hydroxytryptamine, methacholine), assayed by measurement of IP_x responses and/or by Ca^{2+} fluorimetry. Subsequently, we have transfected M_3 muscarinic receptors into Lvec and L15 cells, which are functionally coupled to mobilization of intracellular Ca^{2+}. For example, one M_3-transfected L15 clone expressing 505 ± 7 fmol/mg of M_3 receptor protein (B_{max} by *N*-[^{3}H]methylscopolamine binding) responded to 100 μM methacholine with a peak increase in intracellular Ca^{2+} of 552 ± 60 nM and a plateau of 264 ± 10 nM, from a resting Ca^{2+} concentration of 129 ± 2 nM (R. A. Wilcox and S. R. Nahorski, unpublished work).

DISCUSSION

The present studies reveal that L15 cells stably overexpress the type-1 InsP_3R at levels ~ 8-fold higher than the vector-transfected control Lvec cell line. The InsP_3-binding sites in both cell lines are indistinguishable in terms of affinity and selectivity for various inositol polyphosphates, supporting previous work on these cells [17]. However, the studies here provide new data on: (a) the stability of expression and potential utility of these cells; (b) the subcellular distribution of endogenous and transfected type-1 InsP_3R; and (c) the behaviour of full and partial InsP_3R agonists in Lvec and L15 cells.

The stability and abundance of type-1 InsP_3R overexpressed in L fibroblasts reported here are not typical of other studies. Overexpression of type-3 InsP_3R in an insulinoma cell line has been reported to be unstable, declining to untransfected levels after several cell passages [30]. In NIH-3T3 fibroblasts transfected with either full-length or truncated rat type-1 InsP_3R cDNA, turnover of InsP_3R proteins was increased, such that stable overexpression levels were only 15–30 % above that of the endogenous receptor [31]. In the present study, overexpressed InsP_3R protein levels were considerably higher, suggesting that transfection systems, host cell types and culture conditions may play roles in determining the levels and stability of InsP_3R overexpression. The stability of type-1 InsP_3R overexpression in L15 cells will permit co-transfection of other components of the phosphoinositide pathway, as well as potential modulators of InsP_3R function. Indeed, characterization of M_3 muscarinic receptor-transfected Lvec/L15 cells is currently underway.

Both Lvec and L15 cells express predominantly the type-1 InsP_3R and a very low amount of the type-2 isoform. The type-3 InsP_3R does not appear to be present in these cell lines. L15/Lvec cells are atypical in expressing predominantly one type of InsP_3R, the type-1 isoform, and consequently any functional changes in InsP_3-induced Ca^{2+} release between Lvec and L15 cell-lines are likely to be due to increased expression of the type-1 InsP_3R rather than a switch in the major InsP_3R isoform expressed. Hence, these cell lines may prove useful in evaluating the functional properties of predominantly type-1 homotetrameric InsP_3R populations.

A major question relating to InsP_3R overexpression is the subcellular location of endogenous and introduced proteins. Membrane subfractionation experiments indicated that the type-1 InsP_3R in Lvec and L15 cells is associated with membranes derived from intracellular compartments, in agreement with the immunocytochemical approach reported by Miyawaki et al. [17]. Type-1 InsP_3R in Lvec cells displays a subcellular distribution which mirrors that of the SERCA pump, whereas in L15 cells this association is not as close. However, when expressed as a percentage of the total microsomal anti-(type-1 InsP_3R) immunoreactivity, the majority of the L15 cell type 1 InsP_3R is expressed in membrane fractions bearing the highest density of SERCA protein. Furthermore, an incomplete association between SERCA pumps and endogenously expressed type-1 InsP_3R protein was also observed in SH-SY5Y neuroblastoma membranes, subfractionated using an identical protocol. This indicates that lack of absolute correlation in the distribution of InsP_3Rs and SERCA pumps in L15 cells may be an artifact of cellular disruption and membrane subfractionation, rather than expression of the transfected type-1 InsP_3R in compartments inappropriate for Ca^{2+} uptake and release. This is reflected by the

increased sensitivity of L15 intracellular store Ca^{2+} mobilization to Ins(1,4,5)P_3.

In the report initially characterizing L15/Lvec cells, the EC_{50} for Ins(1,4,5)P_3-mediated Ca^{2+} release from membrane preparations was tenfold lower in L15 than Lvec cells [17] compared with the fourfold difference reported in this study. The Ins(1,4,5)P_3-releasable Ca^{2+} pool size of both cell lines was also considerably smaller than that reported here. Such discrepancies are likely to be due to our use of permeabilized cells rather than membrane fractions [19]. The present study also revealed that both Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 display a two- to four-fold increase in potency of Ca^{2+} release in saponin-permeabilized L15 compared with Lvec cells in steady-state $^{45}Ca^{2+}$-flux assays. This increase in potency was observed using metabolically resistant inositol polyphosphate analogues, including the novel adenophostin A analogue 2-hydroxyethyl-α-D-glucopyranoside 2,3′,4′-trisphosphate and the Ins(1,3,4,5)P_4 analogue 3PS-Ins(1,4,5)P_3, or using Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 at 4 °C, indicating that this effect is not due to differences in inositol phosphate metabolism between the two cell lines. This indicates that changes in the sensitivity of Ca^{2+} release to inositol polyphosphates are due to the functional overexpression of type-1 InsP_3R channels. The difference in EC_{50} values for Ins(1,3,4,5)P_4-induced Ca^{2+} release between L15 and Lvec cells parallels that observed for Ins(1,4,5)P_3, lending support to the notion that Ins(1,3,4,5)P_4 acts as a weak agonist at the InsP_3R [25,32].

The increased sensitivity of Ins(1,4,5)P_3-induced Ca^{2+} release in L15 compared with Lvec cells contrasts with observations made in Purkinje cells, which express high levels of type-1 InsP_3R, and peripheral tissues, which express much lower levels of this channel [2]. However, it is important to note that there are differences in the assays utilized to monitor Ca^{2+} mobilization, (our end-point $^{45}Ca^{2+}$-release assays on suspension-permeabilized Lvec/L15 cells compared with fluorimetric/electrophysiological techniques using flash photolysis of caged InsP_3 in intact Purkinje neurons/non-neuronal cells). Furthermore Purkinje neurons express approximately 10 times higher levels of type-1 InsP_3R than L15 cells.

A maximally effective concentration of 6-deoxy-inositol-(1,4,5)PS_3 in steady-state $^{45}Ca^{2+}$ assays released a smaller proportion of the intracellular Ca^{2+} pool in Lvec cells than a maximal dose of Ins(1,4,5)P_3, as expected for an InsP_3R partial agonist [21]. However, the analogue did not display a markedly enhanced maximal response in L15 cells, which might have been anticipated from classical receptor theory. However, the actions of InsP_3R partial agonists may not depend simply on receptor density, but rather on the intrinsic properties of the receptor itself, or on modulators of InsP_3R channel activity. Overexpression of the type-1 InsP_3R may alter its interaction with modulatory proteins, by virtue of an increase in the ion channel to regulator molar ratio. Levels of expression of other ion channels have been reported to influence their pharmacological and electrophysiological properties. Density-related modifications of channel electrophysiology and pharmacology have been reported for *Xenopus* oocytes microinjected with different amounts of cRNA encoding the Kv1.2 delayed-rectifier K^+ channel [33] or the glycine receptor α_1 subunit [34]. It has been suggested that such changes are a consequence of receptor packing at high densities, resulting in interactions between single-channel complexes and it is possible that inter-channel communication may also occur between InsP_3Rs [35]. Such interactions could determine some of the functional characteristics of the InsP_3Rs overexpressed in L15 cells.

A variety of studies indicate that InsP_3-induced Ca^{2+} release displays biphasic kinetics (for a review see ref. [36]). By analogy with the acetylcholine receptor/channel [37], these two phases may represent different conformational states of the receptor, which correspond to different affinity states in terms of ligand-binding sites and different channel-conductance states. Indeed, hepatic InsP_3Rs are reported to exist in two different affinity states for InsP_3: a low-affinity state in which the channel is active, and a high-affinity state in which it is inactive [38]. Furthermore single-channel studies on purified reconstituted InsP_3Rs have revealed the existence of multiple-channel conductance states [39], which may be favoured by interaction with partial agonists. For example, the fungal metabolite adenophostin B, an InsP_3R agonist, mediates Ca^{2+} release via purified type-1 InsP_3Rs with a high positive co-operativity, whereas InsP_3-induced channel activation only displays modest positive co-operativity [40]. Use of Lvec and L15 cells to study the kinetics of InsP_3R-mediated Ca^{2+} release, in combination with pharmacological tools which may favour certain conductance states of the receptor, will prove useful in elucidating the mechanism of type-1 InsP_3R channel gating.

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